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*Short Communication*

# Promotion of Acetylene Reduction by *Rhizobium*-Soybean Cell Associations *in Vitro*[1]

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## ABSTRACT

**Nitrogenase activity, determined by the acetylene reduction assay, in soybean cell suspensions infected with *Rhizobium*, was stimulated significantly by the addition of succinic acid or glutamine. Acetylene-dependent ethylene values as high as 4.6 μmoles of ethylene/gram dry weight·hour were observed.**

The observation that soybean cells can associate *in vitro* with *Rhizobium japonicum* to form a presumed nitrogenase enzyme complex (4) has been confirmed recently by two independent laboratories using different techniques (1, 6). Although there is one report of $^{15}N_2$ reduction in this system (2), most workers have used the sensitive acetylene reduction assay (3) to monitor the low levels of apparent nitrogenase activity. Attempts to relate *in vitro* rates of acetylene reduction in *R. japonicum*-soybean cell associations to *in vivo* values have suggested that the *in vitro* symbiosis reduces no more than 1% as much acetylene as roots nodulated by the same strain of bacteria (1, 4). Obviously, nitrogenase activity must be increased dramatically if this *in vitro* system is to fulfill its exciting potential. Other workers have reported preliminary evidence suggesting that the *in vitro* system is nitrogen-limited (1), and more extensive studies in this laboratory (7) support that conclusion. The present paper reports that succinic acid produces a 16-fold increase over the previously reported (1) maximum values for nitrogenase activity in this system.

## MATERIALS AND METHODS

Soybean cell suspensions obtained originally from the root of *Glycine max* cv. Acme were cultured and infected with *Rhizobium* strain 32H1 from the cowpea cross-inoculation group as described previously (6). After 7 days of infection in a liquid medium the cultures were plated onto the SCN medium containing 1% agar (6). In the present experiments succinic acid, α-ketoglutarate (potassium salt), *cis*-oxalacetic acid, pyruvic acid, and L-glutamine were added to the SCN medium prior to adjusting to pH 6.0 and autoclaving. Nitrogenase activity was measured with the acetylene reduction assay 2 weeks after plating the infected cells onto the agar medium.

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## RESULTS AND DISCUSSION

Data in Figure 1 reveal that the addition of either succinic acid or α-ketoglutarate can increase acetylene-dependent ethylene production over the values observed on the unsupplemented SCN medium. Pyruvic acid and *cis*-oxaloacetic acid produced a modest response similar to that observed with α-ketoglutarate. Uninfected soybean cell cultures produced no ethylene in either the presence or absence of acetylene. Ethylene was detected in the infected cultures only when acetylene was present. Maximum nitrogenase activity was observed in the presence of 40 mM succinic acid. The mean value at this concentration in Figure 1 is 84.3 μmoles of ethylene/g dry weight·24 hr, which represents a 35-fold increase over controls lacking succinic acid. A maximum rate recorded with 40 mM succinic acid in one experiment was 4.6 μmoles of ethylene/g dry weight·hr, which compares favorably with the maximum of 0.275 μmoles of ethylene/g dry weight·hr previously reported *in vitro* by Child and LaRue (1).

Glutamine recently has been shown to promote nitrogenase activity in the *Rhizobium*-soybean cell association (7). When glutamine and succinic acid are combined in this system, two types of results have been observed (Table I). In experiment 1 (Table I), the combination of 40 mM succinic acid and 10 mM glutamine produced a synergistic increase in acetylene reduction compared with either compound supplied separately. Experiment 2, however, revealed neither a synergism nor even an additive effect from these two substances. The data in experiments 1 and 2 are not necessarily contradictory if they indicate that an unknown factor limited nitrogenase activity to approximately 22 μmoles of ethylene/g dry weight·24 hr. Results similar to those shown for both experiments 1 and 2 in Table I have been observed in other trials. As yet, however, no limiting factor has been identified.

A straightforward interpretation of these data appears most appropriate. ATP is required for nitrogenase activity (5), and succinate supports acetylene reduction by soybean bacteroid suspensions under both aerobic and anaerobic conditions (8). Thus the citric acid cycle intermediates may promote nitrogenase activity in the *Rhizobium*-soybean cell association by increasing ATP production. This system is nitrogen-limited on the SCN medium (7), and promotion of nitrogenase activity by glutamine probably is produced by overcoming limitations imposed on nitrogenase synthesis or activity by suboptimum levels of nitrogen.

Variations in nitrogenase activity between experiments in these studies were similar to those reported previously (6). Such fluctuations were independent of the rhizobial strain present. *Rhizobium*-infected cells plated onto SCN medium supple-

mented with succinic acid and glutamine showed less severe changes in nitrogenase activity, and this procedure may help to solve this problem.

It is not possible to make a direct comparison of the nitrogenase activity in the present experiments with that observed in the intact plant because *Rhizobium* 32H1 does not form nodules on soybeans. Other workers, however, reported that greenhouse-grown Acme soybeans nodulated with *R. japonicum* 61A76 produced 15.0 μmoles of ethylene/g dry weight·hr in the presence of acetylene (1). Field-grown Wayne soybeans produce 220 μmoles of ethylene/g fresh weight·24 hr (3). This latter value represents approximately 46 μmoles of ethylene/g dry weight·hr. Thus, the present *in vitro* system exhibits as much as 10% of the nitrogenase activity reported for a typical field-grown soybean plant. Soybean cells inoculated *in vitro* with several strains of *R. japonicum* also exhibited greater nitrogenase activity in the presence of succinic acid or glutamine, but the levels of activity were significantly lower than replicates infected with *Rhizobium* 32H1.

It is hoped that the addition of succinic acid and glutamine to the SCN medium will facilitate future studies of the *in vitro Rhizobium*-soybean cell association. Many important questions relating to the genetics and biochemistry of $N_2$ reduction in legumes have been difficult to study in an unsynchronized population of root nodules on an intact soybean plant. With levels of nitrogenase activity reported here, one can foresee utilizing the *in vitro* system to investigate some of these problems.

Table I. *Effect of Succinic Acid and Glutamine on Acetylene-dependent Ethylene Production by Acme Soybean Cells Infected with Rhizobium 32H1*

Each value represents the mean ± SE from five to 10 replicates. No endogenous ethylene production was detected prior to the supplying of acetylene.

| Medium Additive | Acetylene-dependent Ethylene Production | |
|---|---|---|
| | Experiment 1 | Experiment 2 |
| *mM* | *μmoles/g dry wt·24 hr* | |
| None | 0.16 ± 0.06 | 0.18 ± 0.16 |
| 40 succinic acid | 6.00 ± 1.64 | 21.9 ± 1.91 |
| 10 glutamine | 1.72 ± 0.51 | 21.9 ± 3.19 |
| 40 succinic acid + 10 glutamine | 21.4 ± 2.49 | 19.9 ± 2.26 |

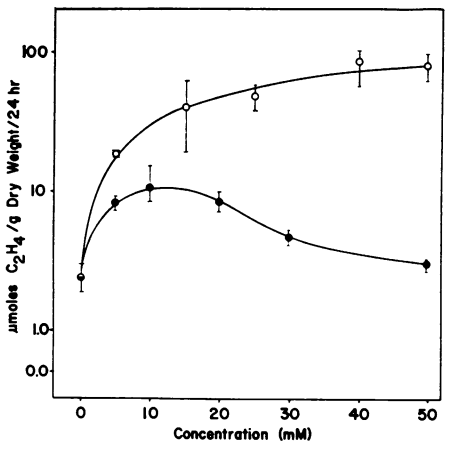


FIG. 1. Acetylene-dependent ethylene production by Acme soybean cells infected with *Rhizobium* 32H1 at various concentrations of succinic acid and potassium α-ketoglutarate. Each point represents the mean ± SE from at least five replicates. No endogenous ethylene production was observed prior to the supplying of acetylene. Replicates containing 10 mM glutamine in the medium without either succinic acid or α-ketoglutarate produced 9.98 ± 2.43 μmoles of ethylene/g dry weight·24 hr. Succinic acid (—○—); potassium α-ketoglutarate (—●—).

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